

Keratin transamidation<sup>☆,☆☆</sup>Jeanette M. Cardamone<sup>\*</sup>

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA

## ARTICLE INFO

## Article history:

Received 21 December 2007

Accepted 13 February 2008

Available online 4 March 2008

## Keywords:

Keratin

Wool

Transglutaminase

Self-crosslinking

Transamidation

## ABSTRACT

Low molecular weight keratin was self-crosslinked by microbial transglutaminase (mTGase) for application to wool fabric. From amino acid determination, keratin produced by the alkaline hydrolysis of wool showed requisite glutamine and lysine required for mTGase-mediated transamidation. Keratin showed less lysyl amine content after combination with mTGase as proof of self-crosslinking. Gel electrophoretic patterns provided evidence of self-crosslinking with the development of relatively higher molecular weight protein bands within 30 min after mTGase was combined with keratin at 30 °C. Increase in the deconvoluted amide II band from IR spectra of keratin after combination with mTGase provided further evidence of transamidation. By examining the ability of keratin to self-crosslink, past findings were elucidated whereby mTGase-mediated crosslinking imparted strength to wool and keratin controlled its dimensional stability. mTGase-catalyzed isopeptide bond formation of keratin to form monosubstituted  $\gamma$ -amides of peptide-bound glutamine in  $\epsilon$ -amino-( $\gamma$ -glutamyl)lysine crosslinks. This system was effective for binding wool to wool, keratin to wool, and keratin to keratin in self-crosslinking.

© 2008 Elsevier B.V. All rights reserved.

## 1. Introduction

In a previous report, when solubilized keratin and microbial transglutaminase (mTGase) were applied to wool fabric after fabric bleaching with an activated peroxide, peroxydicarboximide acid, the dimensional stability of the fabric was controlled to less than 5% area shrinkage [1]. Originally mTGase as an after treatment was found to impart added strength to wool fabric that had been bleached with activated peroxydicarboximide acid and subsequently treated with protease in the novel ARS process for bleaching, biopolishing, and shrinkproofing wool [2,3]. mTGase mediation provided self-crosslinking of wool and crosslinking of solubilized keratin to wool fabric. The purpose of this study is to characterize the interaction of keratin and mTGase.

mTGase (EC 2.3.2.13) catalyzes the transfer of  $\gamma$ -acyl glutamine (Gln) to amine nucleophile, cysteine (Cys), through the formation of a mTGase-Gln transition state complex. mTGase is regenerated as a free enzyme in the presence of a suitable primary amine or it is hydrolyzed [4,5]. In situ mTGase-mediated transamidation of wool proceeds through the formation of isopeptide linkages between keratin's Gln and Lys residues (Fig. 1).

Keratin was used in hydrogels and films as stand-alone products and as a constituent in commercial formulations. Keratin can provide a platform for active agents to deliver topical treatments to woven and non-woven wool fabrics [6–9]. Keratin is extracted from wool by oxidative fracture of cystine (RSSR) disulfide bonds to form sulfonic acid (RSSO<sub>3</sub>H), reduction using mercaptoethanol to form cysteine (RSH) residues, or by some combination of both oxidizing and reducing agents. Strong alkali can decompose aliphatic disulfides by direct attack on sulfur to give thiol (cysteine) and sulfenic acid (cysteic acid) as principal residues. Initial attack of alkali (OH<sup>−</sup>) on cystine (−S−S−) is known to form lanthionine (HOOC−CH(NH<sub>2</sub>)−CH<sub>2</sub>−S−CH<sub>2</sub>−CH(NH<sub>2</sub>)−COOH), cysteinsulfenic acid −NH−CH(CH<sub>2</sub>−SOH)CO−, lysinoalanine, and ornithine alanines formed by the degradation of Cys<sub>2</sub>, Lys, Ser, Thr, and Arg [10–14].

mTGase has been used to link protein, non-protein, and enzymes by covalent bonding through glutamine and lysine amino acids to form films and gels [15,16]. mTGase enzymology is useful for crosslinking soluble proteins and modified protein films, attaching chemical labels or probes to proteins, grafting silk proteins onto wool, and improving wool's properties [17–20].

## 2. Methods

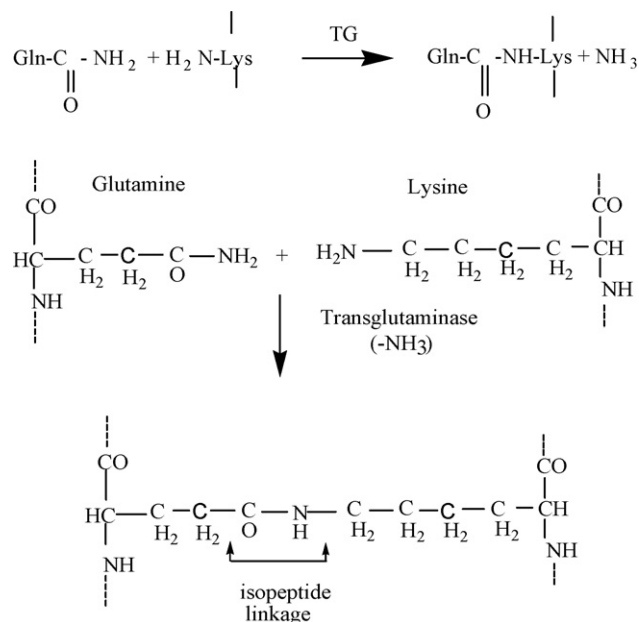
Keratin powder (KP) was produced from the alkaline hydrolysis of wool. Wool fibers, 21  $\mu$ m and 23–24  $\mu$ m, were cryoground to fine wool powders (WP) using a SPEX Cetriprep, Inc. freezer mill (Metuchen, NJ). The powders were immersed in 0.5N NaOH

<sup>☆</sup> Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

<sup>☆☆</sup> US Patent 7,090,701 B2 (15 August 2006).

<sup>\*</sup> Tel.: +1 215 233 6680; fax: +1 215 233 6559.

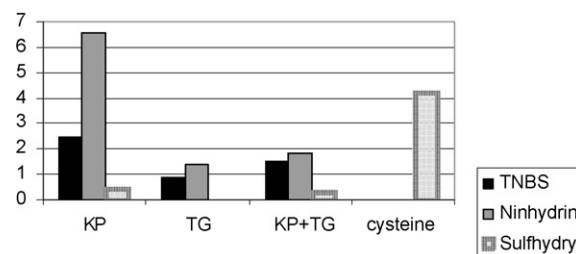
E-mail address: [jan.cardamone@ars.usda.gov](mailto:jan.cardamone@ars.usda.gov).



**Fig. 1.** Isopeptide bond formation of keratin catalyzed by TG to form monosubstituted  $\gamma$ -amides of peptide-bound glutamine in the formation of  $\epsilon$ -amino-( $\gamma$ -glutamyl)lysine crosslinks. Dash-lines denote portions of polypeptide chains in which amino acids are joined by peptide bonds.

solution, pH 13.9, liquor ratio (LR) 50:1 at 60 °C for 3 h and in 0.1N NaOH, pH 12–13, LR 50:1 at 65 °C for 45 h. The hydrolysates were dialyzed through 6000–8000 Da molecular weight cut-off (MWCO) Spectrum Spectra/Por (Thomas Scientific) dialysis tubing and lyophilized under vacuum in a FLEXI-DRY™, FTS Systems, Inc. instrument.

Microbial transglutaminase; *Streptovorticillum mobaraense* isolate, Ca<sup>2+</sup>-independent (ACTIVA™ TG, Ajinomoto Food Ingredients LLC, Ames, IA), is a 38-kDa polypeptide with isoelectric point 8.9 and has cysteine (–SH) as the active center. The Ajinomoto-reported activity of TG is 86–135 units/g, where one enzyme unit is defined as the amount causing the formation of 1  $\mu$ M of hydroxamic acid in 1 min at 37 °C. TG is active at 50–55 °C, pH 6–7, for 10 min and at 30 °C for 35 min [21]. KP was coadded to mTGase for applications to wool.



**Fig. 2.** Colorimetric determinations: lysyl amine from TNBS ( $\times 10^{-3}$  mol/L), free amine from ninhydrin ( $\times 10^{-3}$  mol/L), and sulfhydryl from Ellman's reagent (mM) with reference to cysteine amino acid standard.

## 2.1. Protein assay

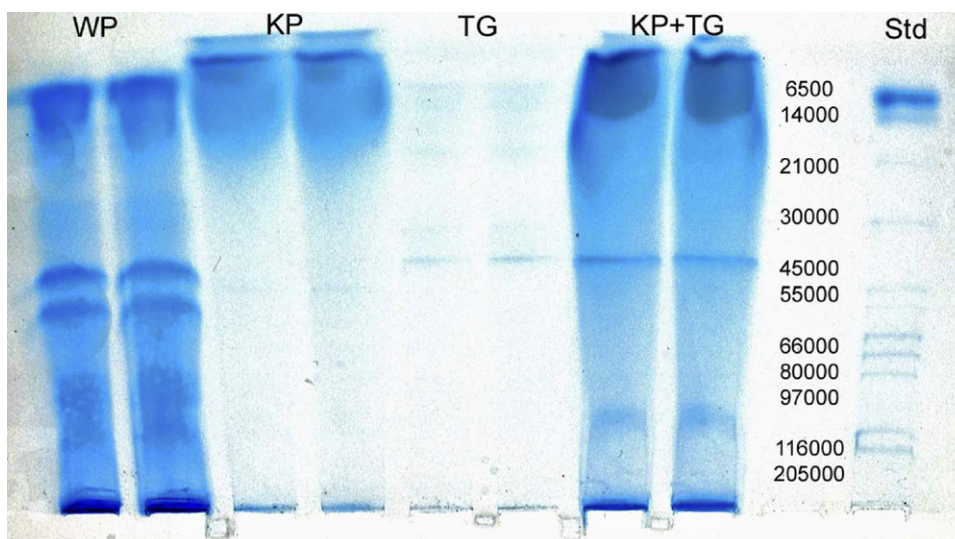
Protein concentration was determined by spectrophotometric analysis on a Varian Cary 50 Conc UV–vis spectrophotometer using Bio-Rad Protein Assay with reference to bovine gamma globulin (BGG) (Bio-Rad Laboratories, Inc.) standard [22,23]. The protein content of solutions of solubilized keratin ranged from 5.1 mg/mL to 5.9 mg/mL.

## 2.2. Amino acid content

Amino acid analysis was performed using a method adapted from Cohen [24]. Briefly, samples were hydrolyzed under vacuum for 20 h in constant-boiling HCl at 112 °C. The hydrolyzed samples were reacted with 6-aminoquinolyl-*N*-hydrosuccinimidyl carbamate to form fluorescent derivatives. Separation was achieved using a 150 mm  $\times$  4.6 mm AccQTag C<sub>18</sub> reversed phase column (Waters Corporation, Milford, MA), eluting with the gradient of acetonitrile and aqueous buffer described as 'system 2' in the reference. Sample peaks were quantified using a fluorescence detector set to excite at 250 nm and detect emission at 395 nm [24].

## 2.3. Lysyl amine groups

Primary lysyl-amines present in mTGase, KP, and in KP + mTGase were detected using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Pierce #28997, Pierce, Rockford, IL) by spectrophotometric analysis at 335 nm using a Varian Cary 50 Conc. UV–vis spectrometer (Fig. 2). There is a color reaction between TNBS and the epsilon:–NH<sub>2</sub> group



**Fig. 3.** Electrophoretic patterns of supernatant from a solution of WP, KP, and 1:1 (w/w) KP+TG lyophilized powder.

of Lys which crosslinks the  $\alpha\text{-NH}_2^-$  group of Glu to form the yellow-colored product, dinitrophenylamino acid whereby  $\text{:NH}_2^-$  displaces the  $\text{-SO}_3^-$  group of TNBS. Decrease in TNBS absorbance signifies a decrease in Lys-amine groups present as shown by Adler-Nissen [25].

## 2.4. Amine groups

### 2.4.1. Free amine

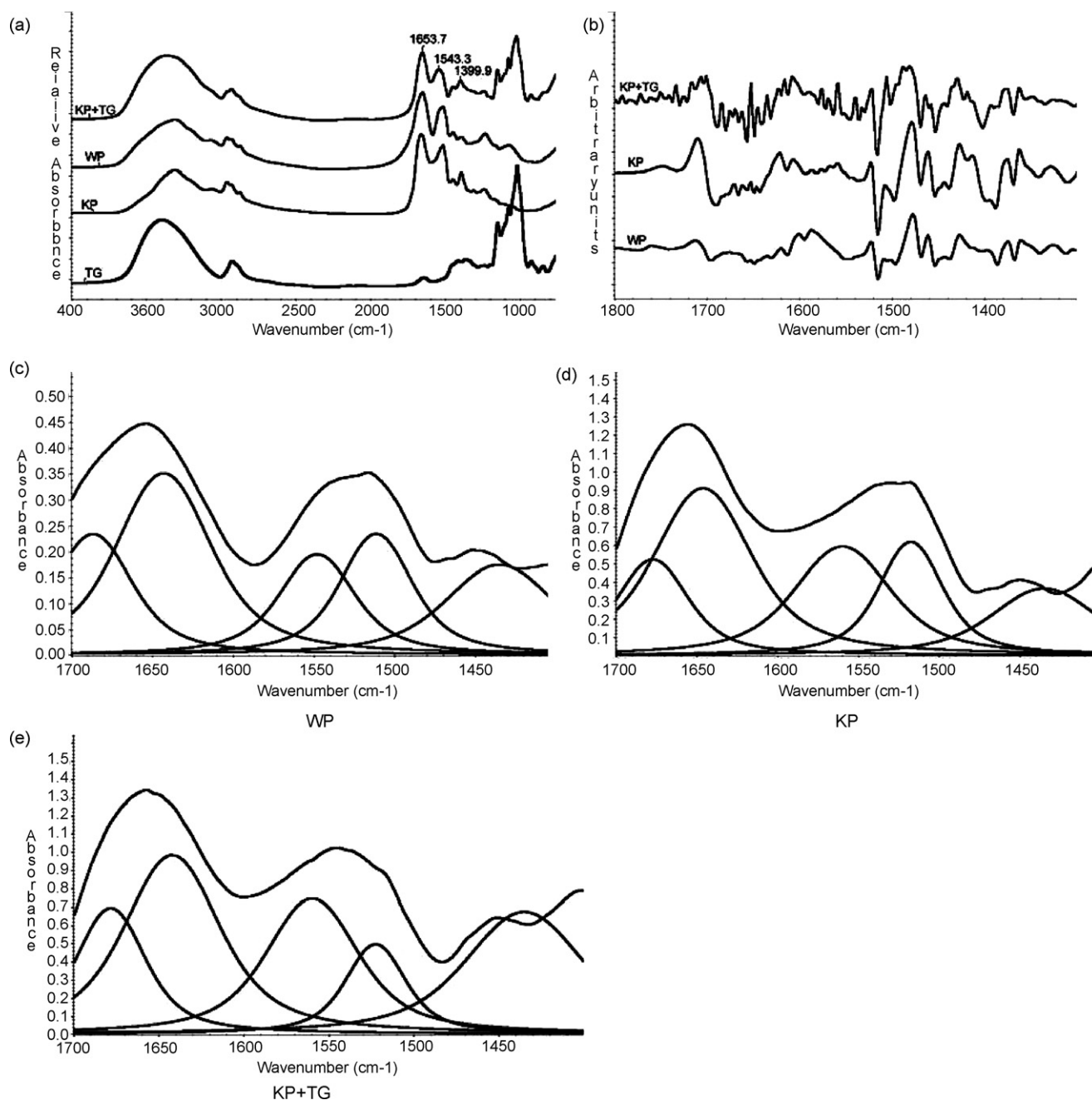
Ninhydrin reagent (Sigma N7285) for detection of primary and secondary amines and amino acids was used to construct a standard curve using lysine as reference for serial dilutions of KP, mTGase, and KP/mTGase to determine their amine content (Fig. 2) [26].

## 2.5. Sulfhydryl ( $\text{-SH}$ ) groups

Ellman's reagent (Pierce #22582) 5,5'-dithio-bis-(2-nitrobenzoic acid) CAS 69-78-3 was used for detection of free sulfhydryl ( $\text{-SH}$ ) by reference to a standard curve generated from serial dilutions of the standard, cysteine hydrochloride monohydrate (Fig. 2) [27].

## 2.6. Gel electrophoresis

Molecular weights (MW) of WP, KP, mTGase, and KP+mTGase were estimated by SDS-PAGE (polyacrylamide gel electrophoresis) using 10–20% Tris-tricine gels in a GeneMate tank (ISC BioEx-



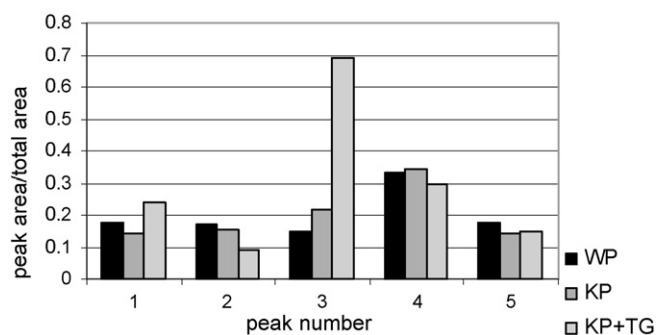
**Fig. 4.** Spectra of WP, KP, TG, and KP+TG: (a) original; (b) second derivative; (c)–(e): deconvoluted amide I, II, and III regions for WP, KP, and KP+TG, respectively.



press) run at 90 V with SDS glycine running buffer and Tris-tricine SDS sample buffer (ISC BioExpress) with reference to molecular weight standards, 6500–20,500 Da (Amersham Pharmacia Biotech UK Ltd.). The soluble proteins, 10  $\mu$ L each of the supernatant fraction of WP from cryogrinding wool fibers, KP (10 mg/mL), mTGase (10 mg/mL), and 1:1 (w/w) KP + mTGase (20 mg/mL) were individually spotted in the gel wells to determine crosslinking of KP by mTGase (Fig. 3).

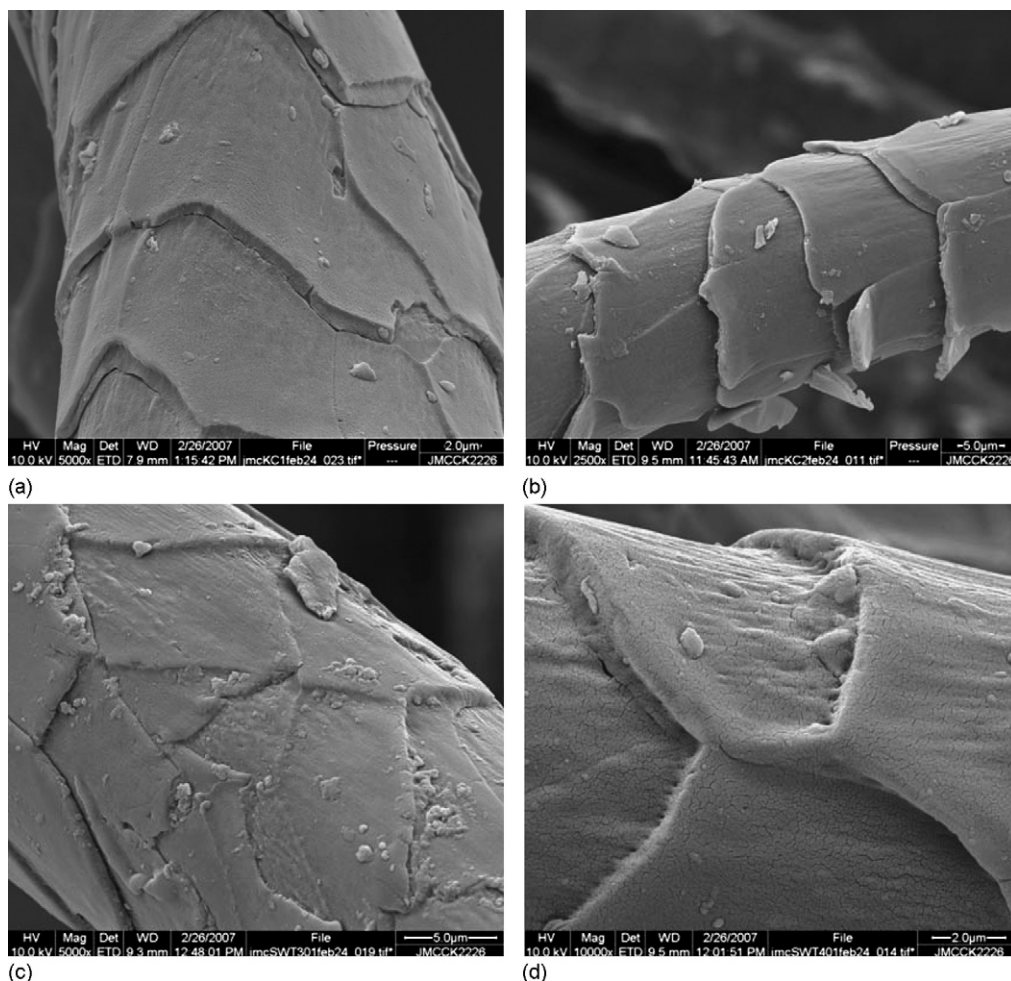
## 2.7. FTIR

WP, KP, mTGase, and KP + mTGase powders were analyzed for changes in the amide spectral region by using a Nicolet Magna FTIR (Thermo Electron Corporation, Madison, WI) equipped with a mercury cadmium telluride detector (MCT/A) and KBr beam splitter. Samples were mixed with KBr, pressed into pellets, and mounted between KBr crystals (Spectra-Tech Inc., Shelton, CT, #0016-034). Spectra were collected with 256 scans against air as background at mirror velocity 1.8988, iris aperture 7, resolution 4, and gain 1 (Fig. 4). The mTGase amide III absorption region overlapped amide III but not amides I and II in (a). Therefore changes in the amide I and II regions were investigated. Omnic 7.3 standard software (Thermo Electron Corporation, Madison, WI) was used in the deconvoluted spectra in (c)–(e) to isolate the individual components of amide I and II bands. The composite Voigt band shapes

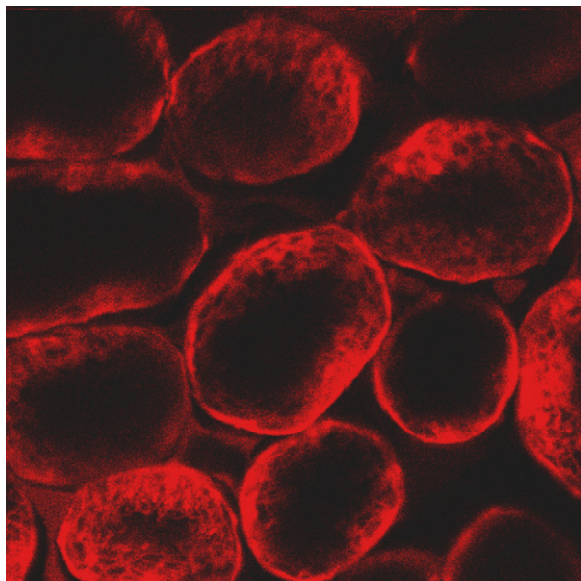


**Fig. 5.** Area ratios of deconvoluted IR spectra in the amide I, II, and III regions: peak area/total area with the following assignments from the deconvoluted spectral regions in Fig. 4c–e: peak 1 ( $1435\text{ cm}^{-1}$ ); peak 2 ( $1511\text{--}1522\text{ cm}^{-1}$ ); peak 3 ( $1548\text{--}1559\text{ cm}^{-1}$ ); peak 4 ( $1642\text{--}1646\text{ cm}^{-1}$ ); peak 5 ( $1677\text{--}1687\text{ cm}^{-1}$ ).

were based on known amide bands commonly used in wool analysis within the  $1700\text{--}1400\text{ cm}^{-1}$  range [28] and were fitted from peaks defined in the second derivative spectrum using no baseline corrections and a peak table consisting of wavenumber values:  $1450 \pm 15\text{ cm}^{-1}$ ;  $1515 \pm 15\text{ cm}^{-1}$ ;  $1545 \pm 15\text{ cm}^{-1}$ ;  $1650 \pm 15\text{ cm}^{-1}$ ; and  $1685 \pm 15\text{ cm}^{-1}$ . In Fig. 5 these peaks are referred to as peaks 1, 2, 3, 4, and 5, respectively.



**Fig. 6.** Wool fibers from wool jersey knit fabric: (a and b) untreated; (c and d) treated with statistically established, optimum conditions of 1:1 (w/w) KP+TG at  $30^\circ\text{C}$  for 30 min.



**Fig. 7.** Confocal image of anionic ARS-bleached wool subsequently treated with KP and dyed with Rhodamine B dye.

### 2.8. Treatment of wool with KP + TG

Wool yarn lengths of untreated wool fiber (Fig. 6a and b), wool fiber treated with KP + TG after ARS bleaching (Fig. 6c and d), 2.5 cm, were withdrawn from untreated and treated fabrics, then glued to aluminum specimen stubs with double-sided SEM tape (Electron Microscopy Sciences, Ft. Washington, PA), and sputter coated with a thin layer of gold. Imaging was performed with a model JSM840A scanning electron microscope (JEOL USA, Peabody, MA) operating at 10 kV in the secondary electron imaging mode and coupled to an Imix-I digital image workstation (Princeton Gamma-tech, Princeton, NJ) [1].

To document anionic surface charge of ARS bleached fibers, Rhodamine B dye (Sigma–Aldrich) was strongly and selectively absorbed by sulfoxylate groups of the wool fiber cuticle (Fig. 7). Fiber cross-sections were embedded in 5:1:1 ethanol:ether:collodion and cross-sections were cut to 5  $\mu$ m thickness on the Micro No. 200-A Microtome (Micro Instrument, Marshfield Hills, MA).

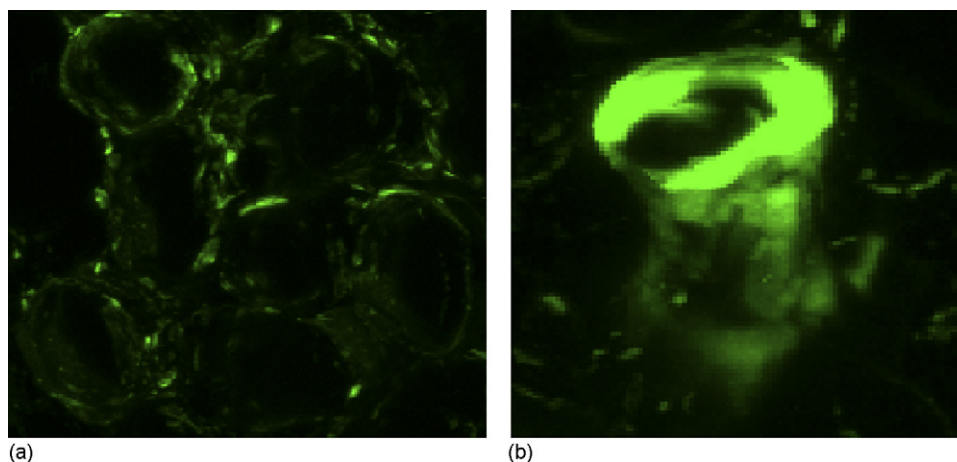
Alexafluor 488 fluorophore (Invitrogen Corporation, Carlsbad, CA) was used to label KP before mixing it with TG for fiber application as a method to tag the location of KP. A Leica TCS Confocal microscope, equipped with an HCX PL40X 1.25 NA and 20 $\times$  air lens with 4 $\times$  digital magnification and excitation, 488 nm and emission, 540–580 nm was used to collect the images in Fig. 8a and b.

### 3. Results and discussion

Wool fiber was treated with NaOH under severe hydrolysis conditions used to solubilize it but not so severe that the protein was destroyed. After hydrolysis of WP to KP, lysine remained constant and glutamine was reported as glutamic acid as dictated by acid hydrolysis used for HPLC amino acid detection (Table 1). Thus glutamine was reported as the sum of glutamine residue and glutamic acid from the HPLC preparation. From the increase in glutamic acid in KP, glutamine was present originally as a viable active site for TG-mediated crosslinking. The method requires that glutamine hydrolyze to glutamic acid and asparagine to aspartic acid. Thus the contents of glutamine and asparagine are really a measure of the sum of the contents of the amino acid residue and its acid form [29–33]. The literature reports that complete hydrolysis of wool by enzymes whereby arginine and glutamine were not hydrolyzed to their acid forms showed a 0.5 ratio of asparagine/aspartic acid and a 1.3 ratio of glutamine/glutamic acid [10]. KP from alkaline hydrolysis showed partial destruction of arginine, serine, threonine, and complete destruction of cystine and tryptophan (not shown), as indicated in Table 1. Alkaline hydrolysis led to hydroxide-ion-catalyzed  $\beta$ -elimination of the disulfide group in cystine [34,35].

TNBS and ninhydrin detection, used to detect lysyl amine, showed loss in the lysyl amine of KP + mTGase, consistent with mTGase-transamidation of KP (Fig. 2). Low amounts of sulfhydryl detected by the Ellman's test are the result of severe alkaline hydrolysis, consistent with minimal amounts of lanthionine formed (undocumented). Low cysteine content was consistent with a loss of cystine to indicate that lanthionine content was not appreciable. Note the detection of amino groups in the KP sample by ninhydrin was appreciably higher than expected when compared to KP amino functionality detected by TNBS. Note that the trinitrophenyl derivative of amino groups can be quantified spectrophotometrically with much greater accuracy than is possible with ninhydrin [36].

The gel electrophoretic pattern for WP was evident from the distinctive bands at 30 kDa, 45 kDa, and 55 kDa and KP was distinguished by a low MW smear covering 6.5–21 kDa. mTGase-



**Fig. 8.** (a and b) Confocal images of wool fibers treated for 30 min at 30  $^{\circ}$ C: Alexafluor 488 labeled KP in KP + TG application.

**Table 1**  
Amino acid composition (mol%) of wool fibers untreated and treated with sodium hydroxide

Amino acid	Untreated wool <sup>a</sup> (WP)	Medium grade wool <sup>b</sup> (WP)	Medium grade wool <sup>c</sup> (KP1)	Fine grade wool <sup>d</sup> (KP2)
Aspartic acid	6.43	7.36	11.2	11.3
Glutamic acid	12.23	13.1	19.3	20.8
Serine	11.0	10.9	3.99	4.0
Glycine	9.44	7.19	6.08	7.0
Histidine	0.80	0.66	0.72	0.80
Arginine	7.30	7.45	5.28	5.16
Threonine	6.38	6.63	2.15	2.47
Alanine	5.76	5.95	7.76	7.91
Proline	7.08	6.88	5.16	6.51
Tyrosine	3.51	3.1	3.15	3.81
Valine	6.38	6.47	7.67	7.57
Methionine	0.59	0.68	0.75	0.84
Cystine <sup>e</sup>	5.65	5.77	0.41	na
Isoleucine	3.45	3.69	4.66	4.82
Leucine	8.08	8.31	12.4	11.2
Phenylalanine	2.93	2.77	2.57	2.80
Lysine	3.01	3.04	3.22	3.03

<sup>a</sup> Reported in Freddi et al. [23].

<sup>b</sup> Medium grade domestic 23–24  $\mu\text{m}$ , 2.5 in. staple wool: not significantly different from fine wool amino acid content (not shown).

<sup>c</sup> KP from medium grade fibers dissolved in 0.1N NaOH, 65 °C, 45 h, dialyzed, and lyophilized.

<sup>d</sup> KP from fine grade 21  $\mu\text{m}$ , 1.5 in. staple length wool fiber dissolved in 0.5N NaOH at 60 °C for 3 h, dialyzed, and lyophilized.

<sup>e</sup> Cysteine plus half cystine.

mediated crosslinking of KP produced KP + mTGase bands from 6.5 kDa to 30 kDa and a relatively high MW band at 110 kDa. The mTGase band at ~40 kDa remained intact.

First derivative spectra (not shown) indicated the differences of KP and KP + mTGase samples in the amide I and II regions. The second derivative spectra in (b) defined the differences among WP, KP, and KP + mTGase in the amide I and II regions. The amide I region (1700–1600  $\text{cm}^{-1}$ ) represents the peptide carbonyl stretching vibration of the CONH unit together with an out-of-phase CN stretching component and a small contribution from the CCN deformation. The amide II region (1600–1435  $\text{cm}^{-1}$ ) represents NH in-plane bending plus CN stretching with C–C stretching, C=O in-plane bending, and NC stretching. The amide III region (1420–1405  $\text{cm}^{-1}$  and 1339–1200  $\text{cm}^{-1}$ ) is associated with NH in-plane bending plus CN stretch plus O=C–N bending. The region indicative of peroxide oxidation is 1040–1175  $\text{cm}^{-1}$ . Sulfonate linkages from cysteic acid residues and cysteine-S-sulfonate (Bunte salt) show a strong asymmetrical stretching vibration at 1022  $\text{cm}^{-1}$  from sulfitolysis cleavage of the cystine disulfide bond to give cysteine thiol and the Bunte salt residue [37,38]. The principal absorption differences were in the amide II region (peak 3) after comparing the ratio of deconvoluted peaks (1–5) to the corresponding total area of all the five peaks for each of the samples: WP, KP, and KP + mTGase (Fig. 5). The increase in amide II band in Fig. 5 (peak 3) provided evidence for KP self-crosslinking because it indicates amidation. The self-crosslinked KP, sample (KP + mTGase), area ratio of amide II/total was appreciably greater than KP's.

Scanning electron micrographs (Fig. 6) show untreated wool fiber (a and b) with intact surface-projecting scales (known to be responsible for shrinkage) and wool fiber (c and d) treated with KP + TG after ARS bleaching [1].

In c and d, the ridges at the base of the projecting scales have been filled in with KP and smoothed to prevent projective scales from interlocking. This profile is diagnostic for shrinkproof, dimensionally stable wool fiber.

Confocal microscopy showing anionic charge was retained on the surface of KP-treated ARS bleached fibers dyed with cationic Rhodamine B dye (Fig. 7).

Confocal microscopy fluorescence of KP labeled with Alexafluor 488 fluorophore before mixing it with TG showed that KP was selectively attached to the fiber surface.

#### 4. Conclusions

Keratin powder was obtained from severe alkaline hydrolysis of wool. As previously reported, this relatively low molecular weight keratin, when applied as a coating to wool fabric, controlled shrinkage and retained fabric strength. These studies suggested that TG-mediation led to in situ, solid-state crosslinking in wool fabric so that strength was improved. This study investigated further the potential for TG catalysis to mediate self-crosslinking of KP. Glutamine and lysine residues of KP were involved in TG-transamidation when KP and TG were mixed 1:1 (w/w) at 30 °C for 30 min. Evidently KP's small, water-soluble peptides with 63.3% less serine, 15% less glycine, 29% less arginine, 93% less cystine, 68% less threonine, but no loss in lysine, and measurable glutamine were capable of self-assembly by forming  $\epsilon$ -amino-( $\gamma$ -glutamyl)lysine crosslinks by forming monosubstituted  $\gamma$ -amides of peptide-bound glutamine. TG crosslinking of KP showed 72.5% less lysyl amine in KP + TG. Gel electrophoretic patterns showed a broader MW distribution and a new higher MW band at 110 kDa in KP + TG. Further evidence for KP self-crosslinking was provided by deconvoluted FTIR spectra that revealed an increase in amide II absorption in the product, KP + TG. Self-crosslinked KP in applications of KP + TG provided the coating observed in the images of shrinkproof wool and when applied to bleached wool, the fiber's anionic charge was preserved.

Overall, wool fabric, WP, and KP from wool retain lysines and glutamines, which can be self-linked or crosslinked by covalent bonding through TG-mediation to seal them together: keratin to keratin, keratin to wool, and wool to wool. This work suggests that even low MW keratin can undergo TG-mediated self-crosslinking to form a useful coating for linking substrates and for binding other protein materials. Even relatively small keratin proteins can participate with TG in acyl-transfer reactions to bind body tissue, protein substrates, and a broad array of Gln-Lys carboxamide-active agents as substrate materials.

#### Acknowledgements

The author acknowledges the technical support of Rafael Garcia for amino acid determination and Bun-Hong Lai and William Stannert for experimentation.

## References

- [1] J.M. Cardamone, J.G. Phillips, *Text. Res. J.* 77 (2007) 277–283.
- [2] J.M. Cardamone, *Text. Res. J.* 77 (2007) 214–221.
- [3] J.M. Cardamone, J. Yao, US Patent 7,090,701 B2 (2006).
- [4] A. Case, R.L. Stein, *Biochemistry* (2003) 9466–9481.
- [5] S.I. Chung, R.I. Shrager, J.E. Folk, *J. Biol. Chem.* 245 (1970) 6424–6435.
- [6] P.M.M. Scrooyen, R. Oberthur, US Patent 7,169,896 B2 (2007).
- [7] A.J. Siller-Jackson, M.E. Van Dyke, S.F. Timmons, C.R. Blanchard, R.A. Smith, US Patent 6,544,548 B1 (2003).
- [8] M.E. Van Dyke, US Patent 7,001,987 B2 (2006).
- [9] P.V. Peplow, D. Roddick-Lanzilotta, US Patent 2005/0232963 A1 (2005).
- [10] J.A. Maclaren, B. Milligan, *Wool Science*, Science Press, 1981, pp. 1–17.
- [11] M.J. Horn, H.D.B. Jones, S.J. Ringel, *J. Biol. Chem.* 144 (1942) 93–98.
- [12] D.E. Goddard, L. Michaelis, *J. Biol. Chem.* 106 (1934) 605–614.
- [13] K.Y. Umeda, K. Nadachi, Y. Sakai, M.S. Nogami, US Patent 2005/0232875 A1 (2005).
- [14] R.S. Asquith, J.J. Garcia-Dominguez, *J. Soc. Dyers Color.* 84 (1968) 211–216.
- [15] H. Green, B. Compton, G. Corey, P. Djian, US Patent 6,919,076 B1 (1999).
- [16] M. Griffin, R. Casadio, C.M. Bergamini, *Biochem. J.* 368 (2002) 377–396.
- [17] K. Ikura, M. Goto, M. Yoshikawa, R. Sasaki, H. Chiba, *Agric. Biol. Chem.* 48 (1984) 2347–2354.
- [18] J. Cortez, P.L.R. Bonner, M. Griffin, *Enzyme Microb. Technol.* 34 (2004) 64–72.
- [19] J. Cortez, A. Anghieri, P.L.R. Bonner, M. Griffin, G. Freddi, *Enzyme Microb. Technol.* 40 (2007) 1698–1704.
- [20] G.D. Du, L. Cui, Y. Zhu, J. Chen, *Enzyme Microb. Technol.* 40 (2007) 1753–1757.
- [21] M. Motoki, et al., US Patent 5,156,956 (1992).
- [22] M.M. Bradford, *Anal. Biochem.* 72 (1976) 248–254.
- [23] G. Freddi, M. Tsukada, H.J. Shiozaki, *J. Appl. Polym. Sci.* 71 (1999) 1573–1579.
- [24] S.A. Cohen, K.M. De Antonis, *J. Chromatogr. A* 661 (1/2) (1994) 25–34.
- [25] J. Adler-Nissen, *J. Agric. Food Chem.* 27 (1979) 1256.
- [26] Ninhydrin Reagent, Solution, SIGMA Product Information, St. Louis, MO, IRB/RXR 10/03 (2003).
- [27] Ellman's Reagent, PIERCE Biotechnology, Rockford, IL, 0311W.
- [28] A. Aluigi, M. Zoccola, C. Vineis, C. Tonin, F. Ferrero, M. Canetti, *Int. J. Biol. Macromol.* 41 (2007) 266–273.
- [29] L.A. Holt, B. Milligan, C.M. Roxburgh, *Aust. J. Biol. Sci.* 23 (3) (1971) 509.
- [30] M. Tsao, D.E. Otter, *Anal. Biochem.* 269 (1999) 143–148.
- [31] F.F. Shih, *J. Chromatogr.* 322 (1985) 248–256.
- [32] W.R. Cuthbertson, H. Phillips, *Biochem. J.* 39 (1945) 7–17.
- [33] G. Coward-Kelly, F.K. Agbogbo, M.T. Holtzapple, *Bioresource Technol.* 97 (2006) 1344–1352.
- [34] A.N. Syed, K. Ahmad, U.S. 5,641,477 (1997).
- [35] E. Tolgyesi, F. Fang, in: Orfanos, Montagna, Stüttgen (Eds.), *Hair Research*, Springer-Verlag, Berlin/Heidelberg, 1981, p. 116.
- [36] A.F.S.A. Habeeb, *Anal. Biochem.* 14 (1966) 328–336.
- [37] L.J. Bellamy, *The Infrared Spectra of Complex Molecules*, 3rd ed., Wiley, New York, 1975, p. 407.
- [38] P. Erra, N. Gómez, M.R. Julia, D.M. Lewis, J.H. Willoughby, *Proceedings of the Ninth International Wool Textile Research Conference WRONZ 1995*, vol. II, 1995, pp. 387–393.